Video Article

Chromosome Preparation From Cultured Cells

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Abstract

Chromosome (cytogenetic) analysis is widely used for the detection of chromosome instability. When followed by G-banding and molecular techniques such as fluorescence *in situ* hybridization (FISH), this assay has the powerful ability to analyze individual cells for aberrations that involve gains or losses of portions of the genome and rearrangements involving one or more chromosomes. In humans, chromosome abnormalities occur in approximately 1 per 160 live births[1,2], 60-80% of all miscarriages[3,4], 10% of stillbirths[2,5], 13% of individuals with congenital heart disease[6], 3-6% of infertility cases[2], and in many patients with developmental delay and birth defects[7]. Cytogenetic analysis of malignancy is routinely used by researchers and clinicians, as observations of clonal chromosomal abnormalities have been shown to have both diagnostic and prognostic significance[8,9]. Chromosome isolation is invaluable for gene therapy and stem cell research of organisms including nonhuman primates and rodents[10-13].

Chromosomes can be isolated from cells of live tissues, including blood lymphocytes, skin fibroblasts, amniocytes, placenta, bone marrow, and tumor specimens. Chromosomes are analyzed at the metaphase stage of mitosis, when they are most condensed and therefore more clearly visible. The first step of the chromosome isolation technique involves the disruption of the spindle fibers by incubation with Colcemid, to prevent the cells from proceeding to the subsequent anaphase stage. The cells are then treated with a hypotonic solution and preserved in their swollen state with Carnoy's fixative. The cells are then dropped on to slides and can then be utilized for a variety of procedures. G-banding involves trypsin treatment followed by staining with Giemsa to create characteristic light and dark bands. The same procedure to isolate chromosomes can be used for the preparation of cells for procedures such as fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), and spectral karyotyping (SKY)[14,15].

Video Link

The video component of this article can be found at http://www.jove.com/video/50203/

Introduction

Chromosome analysis is a conventional technique utilized worldwide to diagnose chromosome instability and rearrangements leading to genetic disorders and malignancy[1,2,8,9]. In addition, a higher resolution for the diagnosis and research of constitutional and cancer-acquired genetic abnormalities can be achieved with the combination of the classical cytogenetic procedures and molecular cytogenetic methodologies such as fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), and spectral karyotyping (SKY)[14,15]. More recently, these techniques have been utilized for the evaluation of chromosome instability associated with stem cell research. Karyotypic abnormalities such as aneuploidy of long-term cultured embryonic cells (ES) and adult stem cells of various organisms have been reported by multiple laboratories. Recent evidence supports that some cell lines are inherently more inclined to chromosome instability regardless of culture conditions. For this reason, when establishing and/or maintaining human, mouse, or Rhesus stem cell lines, chromosome analysis is recommended as part of the quality control process. Many reports describe the increasing interest in the use of routine and molecular cytogenetics to monitor the chromosomal stability of stem cells and malignant cells or various organisms in culture[10-13]. These protocols are increasingly being used by nongenetic laboratories for rapid chromosomal assessment of their cultured cells[13]. We present our basic procedures for chromosome preparation from various cell types, which can be applied for both clinical and research purposes and cells derived from various organisms.

Protocol

1. Chromosome Harvesting of Adherent Cells

1. **Standard Protocol**
    1. Grow cells according to specific cell culturing conditions. When the cells have reached logarithmic phase (80% confluency), add 10 µl/ml of Colcemid to the cell culture flask. A minimum of 2 x 10^6 cells is recommended.
    2. Incubate cells at 37 °C in a 5% CO_2 incubator for 45 min. Using a sterile pipette, transfer media from cells into a 15 ml conical tube. Set aside.
    3. Gently wash the cells by adding 2 ml of HBSS Buffer into the flask. Swirl buffer and then remove using a pipette. Discard.

4. Add 1 ml of trypsin, ensuring that it covers the entire surface of the flask. Only leave the cells in trypsin for about 2 min. Once the majority of the cells have detached, pipette the media in the conical tube back onto the cells.
5. Transfer the cell suspension in 10 ml aliquots into 15 ml conical tubes. Centrifuge at 200 x g for 10 min. Remove supernatant and resuspend the pellet.
6. Add 10 ml of 0.075 M KCl which has been prewarmed to 37 °C to the remaining pellet in the conical tube. Vortex tube at medium speed to mix KCl and cells.
7. Incubate cells at 37 °C for 10 min. Centrifuge at 200 x g for 5 min at 25 °C. Remove supernatant (until about 0.5 ml remains) and resuspend pellet.
8. Carefully add 5 ml of fresh Carnoy's Fixative (3:1 ratio of methanol:glacial acetic acid) to the cells while vortexing. Then add 5 ml more of fixative without vortexing for a total of 10 ml.
9. Centrifuge at 200 x g for 5 min. Remove supernatant and resuspend cells. Add 5 ml of fixative to each tube.
10. Centrifuge at 200 x g for 5 min. Remove supernatant and resuspend cells. Add 5 ml of fixative to each tube. The cells can now be stored in 4 °C for up to one year.

2. **Modification to Protocol: Chromosome harvesting of lymphoblastoid cell lines**
    1. Grow cells according to specific cell culturing conditions. (Use flask appropriate for the amount of cells being cultured). When the cells have reached logarithmic phase (approximately 2 days after cells are split), add 10 µl/ml of Colcemid to the cell culture flask.

3. **Modification to Protocol: Chromosome harvesting from whole blood**

    Phytohemagglutinin (PHA), a lectin derived from the red kidney bean, is a powerful mitogen for human T-cells[16]. 72 hr after the addition of PHA to the culture, about 45% of cells are in S phase. This represents the peak mitotic activity, and is the optimum point at which to harvest for chromosome studies. Other mitogens such as pokeweed (1-10 µg/ml) may be used when analyzing B cells[17,18].
    1. Collect at least 1 ml of whole blood in a green top sodium heparin tube. Use within 3 days after collection. Store blood at RT until ready to use.
    2. Aliquot 0.25 ml of whole blood in 10 ml of complete RPMI media containing L-glutamine (20% fetal bovine serum, 1% Penicillin/streptomycin, 1% fungizone, and 1% PHA). Culture at 37 °C with 5% CO_2.

4. **Modification to Protocol: Chromosome harvesting from bone marrow**

    Cytogenetic analysis on bone marrow is helpful in many malignant hematologic disorders as the observation of a chromosomally abnormal clone may be diagnostic for a specific type of leukemia. Chromosome studies are also used to assess disease progression such as the onset of blast crisis and response to treatment. Since bone marrow cells are actively dividing, no mitogen stimulation is necessary[9,19]. For acute leukemias 24 and 48 hr cultures are also set up.
    1. Collect at least 1 ml of bone marrow in a green top sodium heparin tube. Aliquot 0.25 ml of bone marrow in 10 ml of complete RPMI media containing L-glutamine (20% fetal bovine serum, 1% Penicillin/streptomycin, 1% fungizone). Culture at 37 °C with 5% CO_2.

2. Slide Preparation and Solid Staining

A rapid evaluation of one representative slide will provide information on the quality of the harvest before continuing to further procedures such as G-banding, FISH, CGH, or SKY. Some laboratories prefer to solid stain the cells for rapid harvesting and chromosome assessment. Alternatively, a phase contrast microscope may be used for this analysis. Slides are best prepared when the humidity is approximately 50% and the temperature ambient (20-25 °C).

1. Centrifuge the cells at 200 x g for 5 min at 25 °C. Remove the supernatant until only 0.3-0.5 ml remains.
2. After gently resuspending the pellet, pipette three drops of the cell suspension from a distance of about 2 in onto a slide which is tilted at an angle of about 45° and allow the suspension to roll across the slide. Add one large drop of fresh Carnoy's Fixative to the slide.
3. Dry the back of the slide on a paper towel and then sit the slide out to dry for at least 10 min. The slide should be completely dry.
4. Prepare fresh Giemsa Staining Solution (3:1 ratio of Gurr Buffer and Giemsa Stain). Place the slides on a staining rack. Cover the entire slide in the Giemsa staining solution. Let the slides remain in the staining solution for 5 min. Rinse slides with distilled water, drain, and allow to air dry.
5. Add 4 drops of Permount and a cover slip to the slide. Make sure there are no bubbles under the coverslip. The excess Permount can be removed with a paper towel.
6. Analyze cells with a light microscope under 10X and 100X magnification. If the metaphase cells are abundant and well spread, the remaining slides can be used for other experimental procedures.

3. G-Banding Using Trypsin and Giemsa (GTG)

Trypsin is a proteolytic enzyme which denatures euchromatic histones in DNA regions with higher transcriptional activity resulting. Following Giemsa staining, these regions will appear as light bands. Highly condensed chromatin with little or no transcriptional activity (heterochromatin) will have a large portion of its histones protected from the trypsin and will therefore stain darkly following Giemsa staining. It is essential to initially G-band one slide to monitor the conditions and adjust the trypsin timing if necessary for the subsequent slides.

1. Add the following solutions to 4 Coplin jars.

    Jar #1= 30 ml of 1x HBSS and 4 ml of 10x trypsin (0.5%)

    Jar #2= 50 ml of 1x HBSS

Jar #3= 45 ml of 1x HBSS and 5 ml of fetal bovine serum

Jar #4= 50 ml of 1x HBSS

2. Immerse each slide in Jar #1 for 5 sec, quick rinse in Jar #2, leave each slide in Jar #3 for at least 30 sec, quick rinse in Jar #4 then allow slides to dry.
3. Prepare fresh Giemsa Staining Solution (3:1 ratio of Gurr Buffer and Giemsa Stain). Place the slides on a staining rack. Cover the entire slide in the Giemsa staining solution. Let the slides remain in the staining solution for 5 min.
4. Rinse slides in distilled water in the same order that they were stained. Allow the slides to dry for about 10 min. The slide should be completely dry.
5. Add 4 drops of Permount and a cover slip to the slide. Make sure there are no bubbles under the coverslip. The excess Permount can be removed with a paper towel.
6. Analyze cells with a light microscope under 10X and 100X magnification. Adjust trypsin timing of the rest of the slides based on the results of the first slide.

Representative Results

High quality metaphase spreads are essential for chromosome analysis. A successful assay yields chromosomes which are well spread and of suitable chromosome morphology. Properly G-banded chromosomes contain the characteristic light and dark banding patterns.

Figure 1. A successful chromosomal spread in which the chromosomes are of average length, well spread and easily discernible from one another, and sufficient contrast between light and dark bands. Good chromosome morphology allows for the identification of individual chromosomes and evaluation for any rearrangements indicative of chromosome instability.

Discussion

We have utilized the present procedure for chromosome isolation from cells of various organisms, including various cell types obtained from human, Rhesus macaques, rats, and mice[11,20-22]. The standard protocol is provided, but certain key steps and variables may need to be adjusted for these diverse types of cells. Several specific steps are crucial in both the chromosomal preparation and G-banding to ensure the best possible quality of the results. One of the variables which can affect the assay is the Colcemid incubation time. Insufficient time in Colcemid yields fewer metaphase spreads and longer, overlapped chromosomes. Longer incubation times in Colcemid will result in shorter and thicker chromosomes which are difficult to analyze. Another important variable is the molarity of the hypotonic solution. A 0.075 M potassium chloride solution will swell the cells just enough to yield proper chromosome spreading, without lysing the cells. While adding the Carnoy's Fixative solution, the first 5 ml must be added to the pellet while mixing. This ensures that all of the extra proteins are removed before storing the samples

or preparing slides. If in the case that the cells are not mixed well enough, a yellow protein cap will form on top of the pellet, which can cause complications during subsequent procedures.

Proper slide preparation is essential. While dropping the resuspended cell pellet onto slides, make sure that the slide is tilted to approximately 45° angle and there is enough distance (at least 2 inches) from the dropper to the slide so that the chromosomes can properly disperse onto the slide for analysis. Flooding the slide with fixative immediately following dropping the cells on to the slide will also help chromosomes to spread. Temperature and humidity, which affect how fast the cell suspension dries on to the slide, are other factors which affect chromosome spreading. These can be controlled by preparing slides in an environment with approximately 50% humidity and a temperature of about 20-25 °C, and/or by placing slides on a slide warmer for faster drying. For G-Banding, the main factor that affects the quality of the chromosomes is the trypsin exposure times. With a longer trypsin exposure, chromosomes may appear diffused and swollen. Conversely, an inadequately short trypsin incubation will yield chromosomes with indistinguishable bands and little contrast. The trypsin incubation timing is subject to change depending on each specific cell line and harvesting conditions. Therefore, a representative G-banded slide should be first prepared to evaluate the trypsin conditions before staining the rest of the slides. Fetal bovine serum is used to inactivate the trypsin activity prior to staining. We provide the protocol using Giemsa (GTG), but Wright's stain may be used instead of Giemsa for GTW banding and yields similar results.

This procedure is relatively inexpensive and effective to perform. G-banding can be used to diagnose chromosome abnormalities such as translocations, deletions, and aneuploidy which are commonly seen in malignancies, genetic disorders, and stem cells cultured *in vitro*[10,11,13]. This provides the indispensable visualization of the chromosome constitution and the global evaluation of the entire genome in multiple cells. It is commonly used in clinical and research laboratories worldwide for the diagnosis, prognosis, and therapeutic evaluation of cancer cells[10]. Prenatal and postnatal tissue samples are routinely evaluated for the identification of numerical and structural abnormalities which cause genetic disorders such as Down syndrome. Stem cells can be rapidly evaluated for the presence of chromosome instability. However, the resolution of G-banding is limited for the identification of microdeletions or complex chromosome abnormalities as seen in metastatic malignancies.

Therefore, when routine chromosome G-banding is supplemented with procedures such as fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), and spectral karyotyping (SKY), detection rates of chromosomal abnormalities are increased dramatically[23]. For this reason, the utilization of the present protocol in combination with these other molecular cytogenetic procedures is increasingly being used by different laboratories for the evaluation of chromosome instability in both the clinical and research setting[13-15].

Disclosures

The authors have nothing to disclose.

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